Design and *In Vivo* Evaluation of an Oral Delivery System for Insulin

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Purpose. To develop an oral controlled release system for insulin.
Methods. The polymer–inhibitor conjugates carboxymethylcellulose (CMC)-Bowman–Birk inhibitor and CMC–elastatinal were homogenized with polycarbophil–cysteine conjugate, insulin, and mannitol, compressed to 2 mg microtablets and enteric coated with a polymethacrylate. The protective effect of this delivery system for insulin towards enzymatic degradation, as well as the release profile, was evaluated *in vitro.* In addition, the effect of the dosage form on glucose levels of diabetic mice was determined.
Results. Tablets containing the CMC–inhibitor conjugates showed a strong protective effect for insulin. Whereas 91.6 ± 7.4% (mean ± SD, n = 3) of insulin in the dosage form without the inhibitor conjugates has been degraded within 3 h of incubation in an artificial intestinal fluid containing physiological concentrations of trypsin, chymotrypsin, and elastase, 49.7 ± 5.5% (mean ± SD, n = 3) of insulin remained stable in the delivery system containing the polymer–inhibitor conjugates. Additionally, polycarbophil–cysteine (PCP–Cys) provides high cohesiveness of the dosage form, due to the formation of inter- as well as intramolecular disulfide bonds within the polymer matrix. According to this, a controlled release of insulin could be achieved over a time period of 10 h. Furthermore, *in vivo* studies in diabetic mice showed a decrease in basal glucose levels of 20% to 40% during a time period of 80 h.
Conclusions. Mucoadhesive polymer–inhibitor conjugates might represent a promising excipient in delivery systems for oral (poly)peptide delivery.

KEY WORDS: controlled release; enzyme inhibitors; insulin degradation; mucoadhesive polymers; oral insulin delivery; protective effect; serine proteases; thiolated polymers.

INTRODUCTION

Since its discovery, insulin remains indispensable in the treatment of diabetes mellitus. However, stress and discomfort of multiple daily injections make insulin-treated diabetic patients often express desire for a more convenient and socially compatible route of insulin administration than subcutaneous injection. Among the many routes proposed for its application, the oral route is the most attractive due to its convenience and high patient compliance. In addition to patient comfort, oral administration of insulin would be the most physiological route, because in this way insulin would be directly channeled from the intestine to the liver, and thus avoid peripheral hyperinsulinemic effects, which are considered to be a very important factor in the development of arteriosclerosis (1,2). However, the bioavailability of insulin following oral administration is normally very low, due to absorption barriers and the vast enzymatic degradation in the gastrointestinal tract. Approaches used to overcome these barriers and thus improve insulin absorption include simultaneous administration of absorption-enhancing compounds (3,4) and protease inhibitors (5–7). However—focusing on the use of enzyme inhibitors—several side effects, such as systemic intoxications, a disturbed digestion of nutritive proteins, and hypertrophy and hyperplasia of the pancreas may result from their co-administration. According to this, the practical use of enzyme inhibitors seems to be quite questionable, if these side effects cannot be excluded *a priori.* A promising strategy to solve this problem and subsequently to increase the bioavailability of insulin may be seen in the development of drug delivery systems with mucoadhesive properties providing a prolonged residence time in the intestine. The therapeutic agent should thereby be directly released to the mucosa. Furthermore, a controlled release over a time period of at least 10 hours should be feasible, since absorption can also take place in the colon, where the transit is comparatively slow. As the drug ideally should be protected from enzymatic degradation at the absorption site, we focused our research work in recent years on the development of mucoadhesive drug delivery systems with covalently-attached enzyme inhibitors (e.g. 8,9). The covalent attachment keeps the inhibitors concentrated on the drug delivery system, thereby avoiding local and systemic toxic side effects. In a previous *in vitro* investigation we found that the polymer–inhibitor conjugates carboxymethylcellulose-Bowman–Birk inhibitor (CMC–BBI) and carboxymethylcellulose–elastatinal (CMC–Ela) showed a strong protective effect for insulin towards enzymatic degradation by the luminal endopeptidases trypsin, chymotrypsin, and elastase (10). The purpose of the present study was to develop an insulin delivery system based on these inhibitor conjugates in combination with the novel mucoadhesive polymer polycarbophil–cysteine (PCP–Cys), providing a high stability of the dosage form (11). The system was characterized with regard to its release profile and protective effect towards enzymatic attack *in vitro.* Furthermore, we also investigated its potential on diabetic mice *in vivo.*

MATERIALS AND METHODS

Synthesis of the Polymer–Inhibitor Conjugates

The polymer–inhibitor conjugates CMC–BBI and CMC–Ela were synthesized according to the method previously described by our research group (10). The Bowman–Birk inhibitor (BBI; Sigma, St. Louis, MO) was thereby directly linked to the polymer (Na-CMC; Kwizda, Vienna, Austria) via a condensation reaction mediated by 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride (EDAC) (Sigma, St. Louis, MO), whereas elastatinal was first coupled with the

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ABBREVIATIONS: CMC, carboxymethylcellulose; BBI, Bowman–Birk inhibitor; Ela, elastatinal; EDAC, 1-ethyl-3,3-dimethylaminopropyl carbodiimide hydrochloride; Na-PCP, polycarbophil neutralized with NaOH; Cys, cysteine; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline; TNBS, 2,4,6-trinitrobenzenesulfonic acid; TFA, trifluoroacetic acid.

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spacer putrescine dihydrochloride (tetramethylenediamine .2 HCl; Sigma, St. Louis, MO) providing the NH_2 groups for the covalent attachment to the polymer. The resulting elastatinal–spacer conjugate was then immobilized on the polymer. The ratio of polymer to inhibitor was 20:1 (w/w) in both cases. The presumptive structures of the CMC–inhibitor conjugates are shown in Fig. 1

Synthesis of the Polycarbophil–Cysteine Conjugate

Polycarbophil (PCP)–cysteine was synthesized as described previously (12). In brief, one gram of Na–PCP was hydrated in 250 ml of demineralized water. The carboxylic acid moieties of the polymer were activated for 45 min with EDAC in a final concentration of 50 mM. Thereafter, two grams of L-cysteine hydrochloride monohydrate (Sigma-Aldrich, Steinheim, Germany) were added to this reaction mixture. In order to avoid an oxidation of the sulfhydryl groups, the pH value was adjusted to 5. The reaction mixture was then incubated for 3 h at room temperature under permanent stirring. The conjugate was isolated by dialyzing at 10°C in the dark against 1 mM HCI containing 2 μM ethylenediaminetetraacetic acid (EDTA) two times against the same medium but containing 1% NaCl, and finally exhaustively against 0.5 mM HCI. Thereafter, the dialyzed conjugate was precipitated by pouring the gel in a four-fold excess of methanol under permanent stirring. The pH value was thereby adjusted to pH 5–6 by adding 5 M NaOH. The suspension was centrifuged (9000 × g; 10 min) and the precipitate was air-dried and then pulverized in a mortar. A polymer being prepared and isolated in exactly the same way as the polycarbophil–cysteine conjugate but omitting EDAC during the coupling reaction served as control. The polymer–cysteine conjugate was stored at 4°C until further use.

The degree of modification was determined by quantifying the amount of thiol moieties on the polymer via iodometric titration (1 mM iodine; indicator: starch) at pH 3.0 (13). The presumptive structure of the PCP–Cys conjugate is shown in Fig. 2.

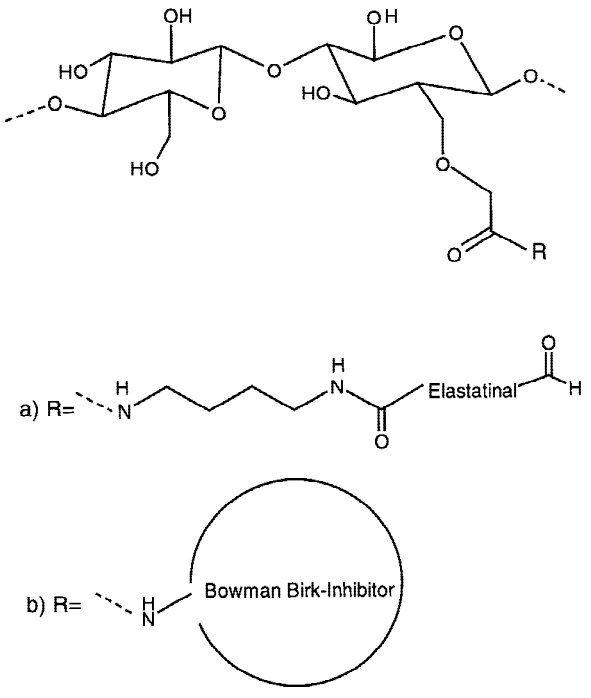


Fig. 1. Schematic presentation of the chemical structure of the CMC–inhibitor conjugates; covalent attachment of the Bowman–Birk inhibitor or elastatinal was achieved by the formation of an amide bond between a carboxylic acid group of the polymer and a primary amino group of the Bowman–Birk inhibitor or the elastatinal–spacer conjugate.

Pre-Treatment of Insulin for Degradation Studies

First, 30 mg of insulin (from bovine pancreas; Sigma, St. Louis, MO) were dissolved in 2.8 ml of 0.1 M NaOH. Then, 200 μl of a $SnCl_2$-solution (0.1 M $SnCl_2$ in 2 M NaOH; Tin(II) chloride, 98%, Sigma-Aldrich, Steinheim, Germany) were added and the reaction was allowed to proceed for 6 h at 40°C under permanent shaking. The resulting insulin fragments were isolated by dialyzing exhaustively against demineralized water for 24 h (dialysis tubing, benzoylated, MW cut-off: 1.2 kD; Sigma, St. Louis, MO). A portion (20μl) of the dialyzed product was directly injected for HPLC analysis in order to verify the fragmentation. Analysis was performed as follows: Nucleosil 100-5C18 column (250 × 4 mm), 40°C; gradient elution: flow rate 1.0 ml/min, 0–22 min; linear gradient from 91% A/9% B to 39% A/61% B (eluent A: 0.1% trifluoroacetic acid in water, eluent B: acetonitrile); detection by absorbance at 220 nm with a diode array absorbance detector (Perkin–Elmer 235C). The dialyzed fragments were then lyophilized by drying the frozen aqueous solution at –30°C and 0.01 mbar (Christ Beta 1-8K; Osterode am Harz, Germany) and stored at –20°C until further use.

Degradation Studies with Carboxypeptidases A and B

First, 5 mg of insulin or insulin fragments were dissolved in 978 μl of a 100 mM phosphate buffered saline (PBS), pH 8. Then, 4.2 units of carboxypeptidase A (EC 3.4.17.1, Type I, from bovine pancreas; Sigma, St. Louis, MO; 4 μl) and 18 units of carboxypeptidase B (EC 3.4.17.2; from porcine pancreas; Sigma, St. Louis, MO; 18 μl of a 1% (w/v) stock solution in 100 mM PBS) were added. The samples were incu-

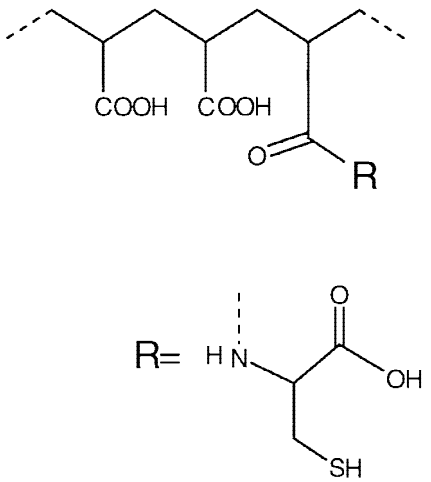


Fig. 2. Structure of the PCP–Cys conjugate; covalent attachment of cysteine was achieved by the formation of an amide bond between a carboxylic acid group of the polymer and the primary amino group of cysteine.

bated for 3 h at 37 ± 0.5°C under permanent shaking. At 30 min intervals aliquots of 50 μl were withdrawn and diluted with the same volume of 20 mM Na_2 EDTA used as stop solution in order to terminate the enzymatic reaction. Thereafter, samples were frozen at –80°C until evaluation. The degree of enzymatic degradation was determined by measuring the increase of free amino acids, detecting their free primary amino groups using 2,4,6-trinitrobenzenesulfonic acid (TNBS) reagent. Samples were thereby incubated with 100 μl of 0.1% TNBS containing 4% $NaHCO_3$ at 37°C for 2 h. Thereafter, 400 μl of demineralized water were added before absorption at 450 nm was measured with a microtitration plate reader (Anthos reader 2001; Salzburg, Austria). The amount of free amino-acid was calculated according to a standard curve of increasing amounts of glycine.

Beside this quantitative evaluation of the degradation, a qualitative evaluation was also performed. Thereby, another 5 mg of insulin or insulin fragments were incubated with carboxypeptidases A and B as described above. After 3 h of incubation, 0.2 ml of 100 mM Na_2EDTA were added in order to terminate the enzymatic reaction. Then, aliquot volumes of 10 μl were withdrawn, and degradation fragments separated by TLC [layer: aluminium sheets Silica gel 60 F_{254} (Merck, Darmstadt, Germany); layer thickness: 0.2 mm; mobile phase: n-butanol/acetic acid/water (3 + 1 + 4); migration distance: 2 × 8.5 cm; detection: spraying with ninhydrin-reagent (0.3 g ninhydrin, 100 ml n-butanol, 3 ml acetic acid)]. Insulin, insulin fragments, alanine, and lysine were used as references.

Preparation of the Insulin Dosage Form

In order to be able to homogenize the polymer–inhibitor conjugates with the model drug and the PCP–cysteine conjugate, the initially lyophilized CMC–inhibitor conjugates were precipitated. Thereby, 20 mg of each conjugate were hydrated in 200 μl of demineralized water and 2.8 ml of isopropanol were added. The precipitates were separated by centrifugation (13500 rpm; 5 min), air-dried, and pulverized in a mortar. Thereafter, proper amounts of insulin, CMC–BBI, CMC–Ela, unmodified CMC, PCP–Cys and mannitol—as listed in Table I—were homogenized and compressed to 2 mg microtablets (diameter: 1.5 mm; depth: 1.0 mm). The hardness of all tablets was thereby kept constant using a standardized compression procedure. After this, tablets were enteric coated with Eudragit L 100-55 (Röhm GmbH, chemische Fabrik; Darmstadt, Germany). Because of their small quantity and size, tablets were not spray coated in a fluidized bed apparatus for economic reasons. Instead, enteric coating was achieved by dipping the tablets five times in a 3% (w/v) acetonic Eudragit L 100-55 solution and air drying.

Table I. Formulations for the Tested Dosage Forms

Components	A (Tablet with insulin and inhibitor-conjugates)	B (Tablet with insulin but without inhibitor-conjugates)	C (Control)
Insulin	0.2 mg	0.2 mg	—
CMC–BBI	0.2 mg	—	0.2 mg
CMC–Ela	0.2 mg	—	0.2 mg
Unmodified CMC	—	0.4 mg	—
PCP–Cys	1.2 mg	1.2 mg	1.2 mg
Mannitol	0.2 mg	0.2 mg	0.4 mg

In Vitro Evaluation of the Drug Release from the Tablets

Because of the small quantities of CMC–inhibitor conjugates available and the fact that a preliminary study showed no difference in the release behavior of tablets containing the inhibitor conjugates or an according amount of unmodified CMC instead, all release studies were carried out with tablets containing 20% unmodified CMC (tablets B, according to Table 1). The *in vitro* release rate of insulin from the drug delivery system was thereby determined by a method which does not conform to the United States Pharmacopeia (USP), due to the small size of the tablets. The dosage forms were placed into 5 ml tubes containing 1 ml release medium. The vessels were closed, placed on an oscillating waterbath (GFL 1092; 60 rpm) and incubated at 37 ± 0.5°C; sink conditions were maintained throughout the study. In the first hour of incubation the release medium was 0.08 N HCl containing 0.2% (m/v) NaCl (liquid 1). Within this time period aliquots of 100 μl were withdrawn every 15 min and replaced with an equal volume of release medium preequilibrated to temperature. Then, the release medium was changed into 1 ml of 100 mM phosphate buffer pH 7.1 (liquid 2). Again, aliquots of 100 μl were withdrawn, but now at 1 h intervals, and replaced with an equal volume of liquid 2 preequilibrated to temperature. The amount of insulin released was evaluated by HPLC analysis as previously described by our research group (10). Concentrations were quantified from integrated peak areas and calculated by interpolation from an according standard curve. Cumulative corrections were made for the previously removed samples in determining the total amount released.

In Vitro Evaluation of the Protective Effect of the Dosage Form Towards Enzymatic Attack

To determine the degree of enzymatic degradation of insulin in the drug delivery system, tablets A and B (according to Table I) were incubated for 3 h with 1 ml of 100 mM phosphate buffer pH 7.1 containing trypsin (704 units/ml), chymotrypsin (4.16 units/ml), and elastase (0.16 units/ml) on an oscillating waterbath (GFL 1092; 60 rpm) at 37 ± 0.5°C. After this, hydrated matrices were withdrawn from the enzyme solution and diluted with 0.5 ml of 0.1% trifluoroacetic acid (TFA) used as stop solution in order to terminate any further enzymatic degradation. The remaining polymer content was then removed by two times centrifugation (20,000 × g, 4°C, Hermle Z 323K). A portion (20 μl) of the supernatant fluid was directly injected for HPLC analysis (series 200 LC; Perkin–Elmer) (10). Insulin and/or degradation products were detected by absorption at 220 nm with a diode array absorbance detector (Perkin–Elmer 235 C). The metabolism of insulin was calculated by following the ratio of the integrated peak area of remaining insulin to the integrated peak areas of degradation products.

In Vivo Evaluation of the Delivery System

Diabetes was induced in thirty B_{alb}/C mice by intraperitoneal administration of 65 mg of streptozotocin per kg (30 mg/ml in 10 mM phosphate buffer, 0.15 N NaCl, pH 7.2;

Sigma, St. Louis, MO). After one week, 30 μl of blood samples were taken from the retrobulbar site and the glucose levels were estimated in the plasma after blood centrifugation using the Trinder glucose kit (Sigma, St. Louis, MO). The glucose levels were determined two times per day for three consecutive days in order to verify the diabetic state. The diabetic mice were divided into three groups of 10 animals and treated separately with the different dosage forms. On the one hand, insulin tablets (A, Table 1) or the control tablets (C, Table I) were administered orally by placing the dosage form very deeply into the throat in order to initiate the swallowing reflex mechanism. Additionally, 50 μl of a 0.2% (w/v) aqueous ascorbic acid solution were administered. On the other hand, insulin was given in ascorbic acid solution [200 μg of insulin dissolved in 50 μl of 0.2% (w/v) aqueous ascorbic acid/mouse]. The dosed mice were kept in restraining cages with free access to water and food. Blood samples of 30 μl were taken periodically from the retrobulbar site and the glucose levels were determined in plasma after blood centrifugation using the Trinder glucose kit. All research using experimental animals adhered to the Principles of Laboratory Animal Care.

Statistical Data Analysis

Statistical data analysis was performed using the Student's *t*-test with $p < 0.05$ as the minimal level of significance.

RESULTS

Characterization of the CMC–Inhibitor Conjugates

The inhibitory potency of various CMC–BBI and CMC–elastatinal conjugates towards the luminally secreted endopeptidases trypsin, chymotrypsin, and elastase has already been evaluated (10). The features of the conjugates described here were in good accordance with them.

Characterization of the PCP–Cys Conjugate

The purified polycarbophil–cysteine conjugate displayed 68.5 ± 8.4 μM (mean ± SD of three experiments) sulfhydryl groups per gram polymer. Omitting the coupling reagent EDAC during the reaction led to a polymer exhibiting a negligible amount of sulfhydryl groups. The stability, swelling behavior, and mucoadhesive properties of various polycarbophil–cysteine conjugates have already been evaluated (11,12). The features of the polymer–cysteine conjugate described here were in good agreement with them.

Degradation Studies with Carboxypeptidases A and B

The TLC studies showed that alanine, representing the carboxy-terminal amino acid in the B-chain of bovine insulin (B30), was the main degradation fragment in both insulin and insulin fragments. Whereas alanine was cleaved to almost 50% from insulin after 3 h of incubation with physiological concentrations of carboxypetidases A and B (14), it was cleaved to nearly 90% from an according amount of insulin fragments, which consist of single A- and B-chains, due to the reduction of inter- as well as intrachain disulfide bonds within the insulin molecule via $SnCl_2$ reagent. Results of the degradation study are shown in Fig. 3.

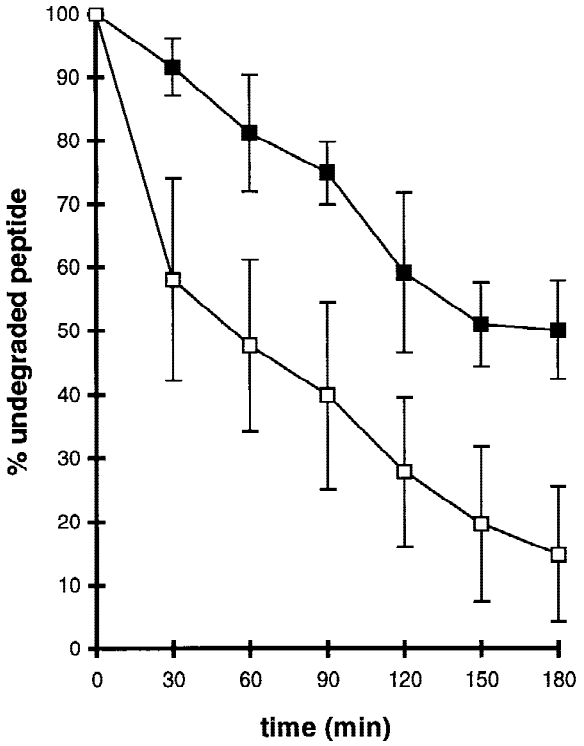


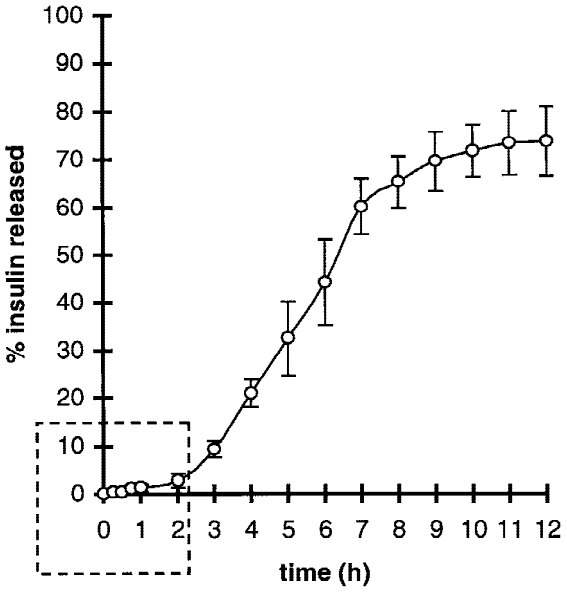
Fig. 3. Degradation of insulin (■) or insulin fragments (□) by a mixture of carboxy-peptidase A (4.2 units/ml) and carboxypeptidase B (18 units/ml) in 100 mM phosphate buffer pH 8, 0.9% NaCl at 37 ± 0.5°C. Each point represents the mean ± SD of at least three experiments.

Release Profile of the Dosage Form

Taking into account an average gastric transit time and a strongly prolonged intestinal transit time due to the mucoadhesive properties of PCP–Cys (12) drug release was investigated in liquid 1 (an artificial gastric fluid, pH 1) between 0 and 1 h, and in liquid 2 (an artificial intestinal fluid, pH 7.1) between 1 and 12 h. As shown in Fig. 4, only a negligible amount of insulin was released in liquid 1, verifying the protective effect of the enteric coating material. In contrast, the release of insulin was markedly increased after changing the pH value to pH 7.1. Due to the high cohesiveness within the polymer matrix, tablets remained stable during the whole time of the study and no disintegration could be observed.

Protective Effect of the Dosage form *In Vitro*

The protective effect of the delivery system towards enzymatic attack of the pancreatic endopeptidases trypsin, chymotrypsin, and elastase was evaluated. Results demonstrated a significant protective effect of the delivery system containing 10% of both CMC–inhibitor conjugates towards the enzymatic degradation of insulin under physiological conditions (14). Whereas the incorporated (poly)peptide drug was almost completely degraded in the dosage form without CMC–BBI and CMC–Ela, approximately 50% of the therapeutic agent remained stable in the delivery system containing the polymer–inhibitor conjugates. Results of this study are shown in Fig. 5.



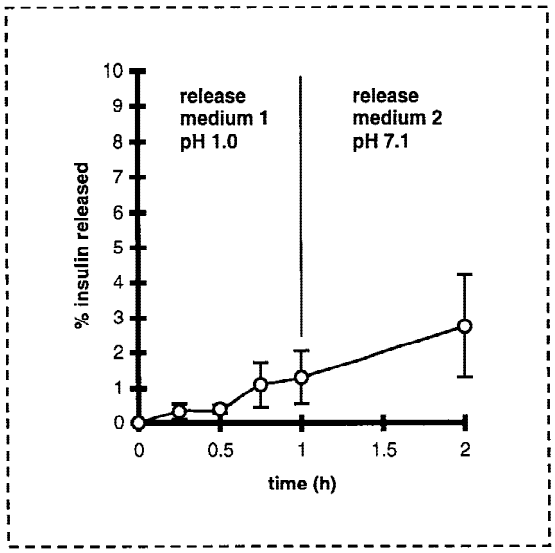


Fig. 4. Release profile of insulin from tablets based on PCP–Cys and unmodified CMC. Studies were carried out in an artificial gastric fluid (release medium 1) for 1 h and then in 100 mM phosphate buffer pH 7.1 (release medium 2) on an oscillating waterbath (60 rpm) at 37 ± 0.5°C. Indicated values are means ± SD of at least three experiments.

In Vivo Evaluation

In order to determine the efficacy of the insulin delivery system, an *in vivo* study was performed, using B_{alb}/C mice as animal model. Although the share of the inhibitor conjugates was only 20% in the delivery system, the bioavailability of orally administered insulin could thereby be significantly improved. As shown in Fig. 6, the basal glucose levels of diabetic mice could be reduced by 20% to 40%. In contrast, tablets without insulin had no effect on the blood glucose level, indicating that the effect is not due to the dosage form itself. Furthermore, the oral administration of insulin in an aqueous ascorbic acid solution (0.2%; w/v) had also no influence on

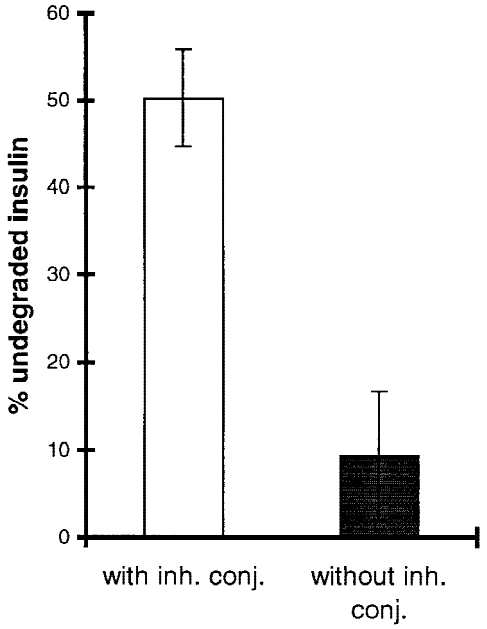


Fig. 5. Amount of remaining undegraded insulin (%) in drug delivery systems with and without 10% CMC–BBI and 10% CMC–Ela conjugate. Tablets were incubated for 3 h in 100 mM phosphate buffer pH 7.1 containing trypsin (704 spectrophotometric BAEE units/ml), chymotrypsin (4.16 BTEE units/ml), and elastase (0.16 succinyl-Ala-Ala-Ala-p-nitroanilide units/ml) at 37 ± 0.5°C. Each bar represents the mean ± SD of three experiments.

the glucose level, which could confirm results of various other *in vivo* studies in mice and rats (e.g. 15).

DISCUSSION

Degradation of insulin is a major factor influencing its therapeutic efficacy after oral dosing. We recently reported that a combination of CMC–BBI and CMC–Ela conjugates show a strong protective effect for insulin towards the luminally-secreted endopeptidases trypsin, chymotrypsin, and elastase *in vitro* (10). Although these enzymes are considered to be mainly responsible for the presystemic metabolism of insulin in the gut (16), exopeptidases may also have an impact on the degradation of insulin. Therefore, one purpose of the present study was to give information about the relative potential of carboxypeptidases A and B to degrade insulin. Taking on the one hand the specificity of these enzymes, and on the other hand the structure of bovine insulin into account, it can be expected, that at least two amino acids of the B-chain—i.e. alanine^{B30} and lysine^{B29}—will be hydrolyzed from the molecule. In contrast, the A-chain of the molecule should remain stable against an enzymatic attack by the luminally-secreted carboxypeptidases. Neither carboxypeptidase A nor B has any specificity for asparagine^{A21} (14), which is the C-terminal amino acid of the A-chain being vicinally located to one of the S-S inter chain bonds. Indeed the results obtained within the present study were in good agreement with these theoretical considerations. Whereas almost 86% of alanine^{B30} were hydrolyzed from free insulin–B-chain, approximately 50% of entire insulin remained stable after 3 h of incubation with physiological concentrations of the exopeptidases. On the one hand, the higher stability of insulin may be due to the

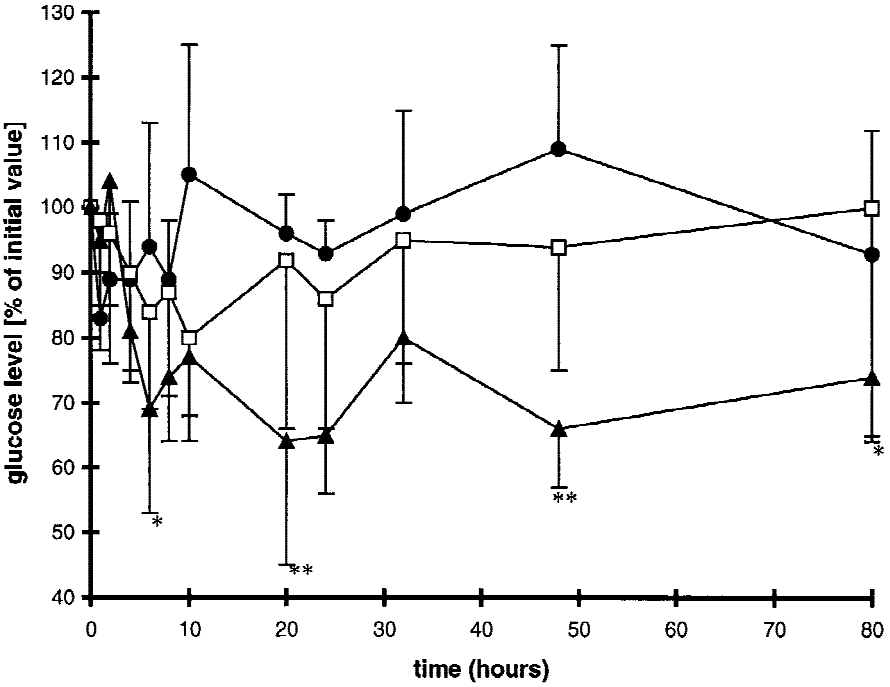


Fig. 6. Effects of orally-administered insulin tablets (A: ▲), control tablets (B: □) and an insulin solution (●) on the blood glucose levels of diabetic B_{alb}/C mice; each point represents the mean ± SD of 10 experiments; *, differs from control $p < 0.05$; **, differs from control $p < 0.001$.

conformational structure of this peptide drug, where the termini of the A- and B-chains can be sterically protected from exopeptidase degradation. The observed stability of insulin may also be explained by the formation of insulin associates and agglomerates (17). On the other hand, pretreatment of insulin with the reductive agent $SnCl_2$ led to fragments which are much more accessible to degradation by exopeptidases.

Focusing on entire insulin, cleavage of B30, representing one of the variable amino acid residues within the different species, may not influence the pharmacological activity of the hormone, especially if one considers that this amino acid is not being involved in the receptor binding (18,19). According to this conclusion, providing a protective effect towards enzymatic attack by luminally-secreted endopeptidases seems to be of higher importance for oral insulin administration. The strong enzyme inhibiting properties of the polymer–inhibitor conjugates CMC–BBI and CMC–Ela have already been mentioned above. Additionally, an appropriate inhibitory activity towards chymotrypsin and even towards carboxypeptidases A and B could be reported recently for the thiolated polymer PCP–Cys (20).

Aside from the protective effect of the inhibitor conjugates, the dosage form itself will also have an important influence on the protection of incorporated peptide drugs. Luminally-secreted proteases must, first of all, penetrate into the polymeric network of the hydrophilic matrix tablet in order to degrade the embedded insulin. Hence, matrix tablets based on mucoadhesive polymers can also provide partial protection towards an enzymatic attack in the intestine. In order to make use of this effect, the cohesiveness of the polymer with the incorporated therapeutic agent should be as high as possible. A rapid disintegration of the delivery system would certainly lead to a strong increase in the surface area of the dosage form, thereby leading to a much higher accessibility of embedded (poly)peptides for intestinal proteases (21). The novel mucoadhesive excipient polycarbophil–cysteine (PCP–Cys) has the capability of forming inter- as well as intramolecular disulfide bonds within the polymeric network (11) and can therefore provide a sufficient stability of the dosage form.

Furthermore, based on reactive thiol groups in its structure, PCP–Cys is covalently bound to the mucus layer due to thiol/disulfide exchange reactions with cysteine-rich mucin glycoproteins. For instance, it could be shown recently, that PCP–Cys conjugates displayed a more than twice as high mucoadhesion *in vitro* compared to unmodified polycarbophil (12). This feature should provide a prolonged residence time of the drug delivery system on the site of adhesion/absorption thereby decreasing the diffusion path of the drug from the delivery system to the absorbing membrane.

Due to the high stability of the carrier matrix, a sustained drug release can be controlled even over a time period of several hours, which would otherwise be impossible if the delivery system disintegrates too early. Dissolution studies demonstrated a controlled drug release over approximately 10 h *in vitro*. However, a plateau phase was reached after 70% of the incorporated drug was set free from the delivery system. As insulin is not covalently bound to PCP–Cys via the formation of disulfide bonds (20), the immobilization of the remaining 30% of insulin in the carrier matrix can only be explained by adsorptive interactions with the polymer.

The blood glucose level of diabetic mice was found to significantly decrease after oral administration of insulin-loaded tablets given as a single dose. The effect appears 4 h after administration and was maintained for approximately 80 h, then reaching the initial value bit by bit. The mechanism of the long lasting effect of insulin-loaded tablets on the blood glucose level cannot be fully explained. The results are nevertheless in good accordance with other similar approaches

(15,22), as the *in vivo* efficacy of these delivery systems also exceeded their *in vitro* release behavior to a great extent. This phenomenon may be due to the role of the liver as regulating organ, where overloads of insulin may be stored and then released continually to related organs (23).

Within the present study we could demonstrate that the oral bioavailability of insulin could be significantly increased if administered as a tablet containing mucoadhesive polymer–inhibitor conjugates fed to diabetic rodents. Above this, the question remains, whether these delivery systems will also have therapeutic benefits to diabetic patients. Therefore, a clearer estimation of the quantity of absorbed insulin is needed, as well as a better understanding of the factors affecting the sustained decrease in the glucose level. Nevertheless, the system described within the present study represents a good starting point in order to make oral insulin delivery feasible in the near future.

CONCLUSIONS

Within the present study a mucoadhesive delivery system has been generated, which might be useful for oral insulin delivery. The combination of polymer–inhibitor conjugates and a thiolated polymer has led to a multifunctional dosage form, displaying following advantages:

- Due to the strongly improved mucoadhesive properties, the residence time of the delivery system on the mucosa should be prolonged, thereby providing a higher absorption rate of the incorporated drug.
- The formation of disulfide bonds within the polymeric carrier system improved the stability and viscoelasticity of the matrix tablets. Because of this feature, a sustained drug release can be controlled even for 10 h, which would be impossible if the dosage form disintegrates too early.
- Thiolated polymers themselves display an inhibitory activity towards luminally-secreted exopeptidases. Due to the combination with polymer–inhibitor conjugates, shielding from the attack of luminally-secreted endopeptidases, a strong protective effect against the most abundant intestinal proteases could be achieved.

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